α-BgTx, EPI or IMI. In contrast the dissociation rate was markedly increased when initiated by MLA compared to α-BgTx, EPI or IMI (Fig 1).

Displacement of [^{125}I]α-BgTx, [^{3}H]EPI and [^{3}H]IMI by MLA was with high potency with an IC_{50} value close to the K_d value of each of these labelled ligands. Displacement of [^{3}H]MLA by α-BgTx, EPI and IMI resulted in low potency, but was characterised by shallow displacement curves.

4 DISCUSSION AND CONCLUSIONS

Saturation studies in *M persicae* membranes demonstrate that [^{125}I]α-BgTx, [^{3}H]EPI, and [^{3}H]IMI all label two binding components of differing affinities in a ratio of 1:3. In each case the sum of the high and low affinity B_{max} values approximated the B_{max} value of the single site labelled by [^{3}H]MLA. [^{3}H]MLA is a novel radioligand which is highly selective in vertebrate nAChRs,[3] but which appears to be unable to distinguish between a heterogeneous population of nicotinic ligand binding sites in insects.

The increase in dissociation rate of [^{3}H]α-BgTx using MLA instead of either α-BgTx, EPI or IMI is consistent with an allosteric interaction by MLA at a binding site other than that of the high affinity [^{3}H]α-BgTx binding site.

Displacement studies have shown that MLA is a potent ligand at sites labelled with high affinity by [^{125}I]α-BgTx, [^{3}H]EPI, and [^{3}H]IMI. The shallow displacement curves obtained when [^{3}H]MLA is displaced by α-BgTx, EPI, and IMI (data not shown) are likely to be due to displacement of [^{3}H]MLA from at least two distinct binding sites, for which the displacing ligands have different affinities, but which are indistinguishable by [^{3}H]MLA.

Interestingly, the high affinity binding of [^{3}H]IMI in hemipteran membranes compared to that in non-hemipteran insects may help explain why IMI is particularly useful for the control of sucking pests.

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Nicotinic acetylcholine receptor chimeras of rat α7 and *Drosophila* SAD reveal species-specific agonist binding regions

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Abstract: Species-specific agonist binding regions of nicotinic acetylcholine receptors (nAChR) were examined. Imidacloprid and physostigmine (Phy) selectively activated insect nAChR composed of *Drosophila* second alpha-like subunit (SAD) and chick β2, in contrast to rat α7 nAChR. The Phy-activated currents were α-bungarotoxin (α-BGT) sensitive, suggesting activation at the agonist binding loop C. Several SAD-α7 chimeras were constructed, by switching agonist binding regions, and expressed in oocytes. Though none of the chimeras was activated by a range of nicotinic agonists, [^{125}I]α-BGT binding revealed homomeric assembly of all chimeric cDNAs. Phy differentially displaced [^{125}I]α-BGT from the nAChR chimeras, suggesting that the β subunit is not involved in Phy binding, and that Phy targets the insect agonist binding loop C.

Keywords: nicotinic receptor; agonist binding; chimera; physostigmine; imidacloprid; acetylcholine receptor; neonicotinoid

1 INTRODUCTION

Multiple nicotinic acetylcholine receptor (nAChR) subtypes exist, both within and between species. Insects express only neuronal nAChR, while, in vertebrates, endplate and neuronal nAChR types are distinguished. A range of different insect and vertebrate α subunits exist, which may be combined with various β subunits to form functional nAChR. Three loops in the α subunits are thought to be involved in acetylcholine (ACh) binding,[1] ie loop A (residues 86–93), loop B (residues 148–151), and loop C (residues 190–198). The latter includes the two adjacent cysteines characteristic of α subunits. Distinct nAChR subtypes are distinguished by their physiological and pharmacological properties. In addition, differential sensitivities to chemical compounds may occur, suggesting a role in species-selective toxicity. The nitroguanidine insecticide imidacloprid selectively activates insect nAChR by binding to the agonist

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binding site.[2,3] The carbamate, physostigmine (Phy), selectively activates whole-cell nicotinic currents in locust neurons and in *Xenopus* oocytes expressing fruit fly nAChR resulting from cDNA injection of the second α-like *Drosophila* (SAD) and the chick β2 subunit.[4] Whole-cell currents during stimulation with Phy could not be detected in various cell types expressing different vertebrate nAChR.[4] Phy appears also to target the nAChR agonist binding site, as Phy-activated currents are sensitive to competitive antagonists.[4] This contrasts with previous reports that Phy binds to a separate site instead of the agonist binding site at vertebrate nAChR.[5] These observations suggest that ACh binding sites of insects differ from those of vertebrates.

We investigated species-specific nAChR agonist binding sites at the cellular and molecular level. Several nAChR chimeras of rat α7 and *Drosophila* SAD were constructed, in which distinct agonist binding domains of α7 were replaced by the corresponding domains of SAD cDNA. Agonist binding and function of rat α7, insect SAD/chick β2, and the chimeras in *Xenopus* oocytes was analysed.

2 METHODS AND RESULTS

Pharmacological properties of rat α7 nAChR and fruit fly nAChR resulting from co-expression of the *Drosophila* SAD α and the chick β2 subunit in *Xenopus* oocytes were investigated, using the dual microelectrode voltage clamp method. ACh activated the wildtype rat α7 and SAD/chick β2 nAChR. Concentration-effect curves revealed EC_{50} values of 179 (±65) μM ($n = 3$) and 15 (±4) μM ($n = 3$), with Hill coefficients of 1.44 (±0.22) and 1.35 (±0.09), respectively, which are in the same range as those reported previously.[6,7] Imidacloprid at 10 μM (data not shown), and 100 μM Phy evoked inward currents in oocytes expressing SAD/chick β2 nAChR (Fig 1A). The Phy-activated current was completely blocked by 50 nM α-bungarotoxin (α-BGT; not shown), suggesting that Phy binds to the agonist binding site of SAD/chick β2 nAChR. Neither Phy (Fig 1A) nor imidacloprid (not shown) activated any current at the rat α7 nAChR.

The binding site for species-selective nAChR activation was determined by construction of chimeras of rat α7 and *Drosophila* SAD cDNA, in which agonist binding regions of α7 were replaced by the corresponding sequence of SAD cDNA. The chimeras were constructed using a two-step PCR amplification. For chimeras 7S.1 and 7S.2 the rat α7 cDNA region His137 to Met226, including ACh binding loop B with the putative Phy-site, and loop C were replaced, whereas in 7S.2 the Phy-site (Lys165) was changed to Gln. 7S.3 and 7S.4 contain the SAD sequence corresponding to α7 His137 to Trp196 and α7 Trp196 to Met226, respectively, including SAD loop C and loop B. The final PCR products were cloned into the HindIII-XbaI sites of pcDNA3, were completely sequenced, and expressed in *Xenopus* oocytes. Neither

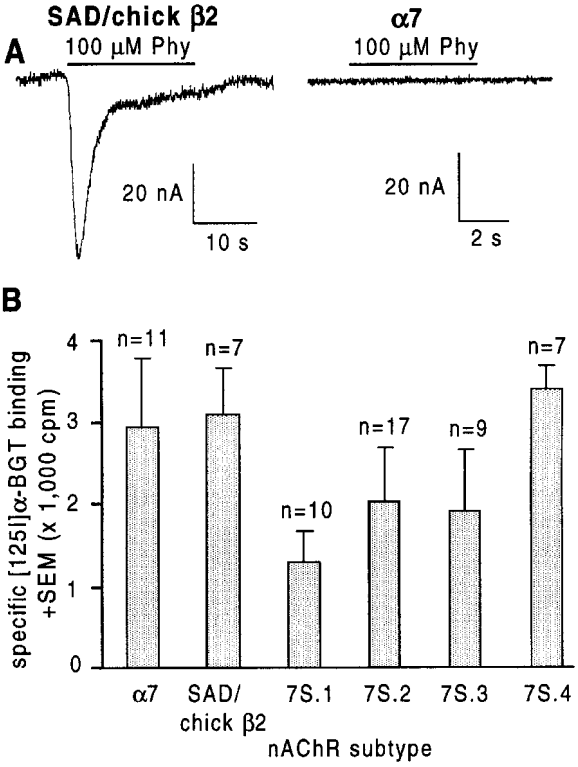

Figure 1. Properties of oocytes expressing SAD/chick β2, α7 and α7-SAD chimeric nAChR. **A**. Phy (bar) induced a nicotinic inward current in oocytes expressing SAD/chick β2 nAChR, in contrast to rat α7 nAChR. Holding potential −80 mV. **B**. Oocytes were preincubated for 60 min in phosphate-buffered saline (PBS), supplemented with 0.1% bovine serum albumin (BSA), incubated for ≥120 min in PBS with 0.1% BSA containing 5 nM [^{125}I]α-BGT. After five washes radioactivity was counted. Non-specific binding in non-transformed oocytes ranged from 9 to 22% of total binding, and was subtracted to calculate specific [^{125}I]α-BGT binding. For all subtypes, specific binding was significantly higher compared to nonspecific binding ($P < 0.001$).

ACh, Phy nor tetramethylammonium (TMA) was able to induce an ion current in any of the chimeras. To examine membrane expression of chimeric nAChR proteins, binding of [^{125}I]α-BGT was analysed in single *Xenopus* oocytes. All chimeric cDNAs were capable of assembly into homomeric nAChR, and bound [^{125}I]α-BGT (Fig 1B).

Subsequently, displacement of [^{125}I]α-BGT by Phy was analysed. Phy at 1 μM displaced 42 (±7)% ($n = 3$) of bound [^{125}I]α-BGT from oocytes expressing 7S.3 (containing SAD loop C) and 55 (±19)% ($n = 4$) from wild-type SAD/chick β2 oocytes. Displacement was less (13 (±47)% ($n = 6$)) from oocytes expressing 7S.4 nAChR (containing SAD loop B with the putative Phy-site).

CONCLUSIONS

The results show that Phy and imidacloprid selectively activate insect nAChR. Further, the nAChR chimeras of the rat α7 and *Drosophila* SAD α subunits successfully assemble into homomeric nAChR proteins, as revealed by [^{125}I]α-BGT binding. Lack of function of the α7-SAD chimeras appears to be due to deficient

coupling of agonist binding to gating of the ion channel, indicating that agonist binding and subsequent ion channel opening are separate, but related processes. Phy differentially displaces [^{125}I]α-BGT from the chimeric nAChR, suggesting that the β subunit is not involved in Phy binding, and that Phy targets the insect agonist binding loop C.

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Imidazenil, a new drug for the management of convulsions in organophosphate intoxication in rodents

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Abstract: The summary deals with the anti-convulsant and antilethal effects of a new benzodiazepine receptor partial agonist, imidazenil, in DFP intoxication. It has been demonstrated that imidazenil (2–5 mg kg^{-1}) significantly decreases convulsion intensity, rapidly inhibits seizure patterns in brain bioelectrical activity and significantly increases the anti-lethal efficacy of atropine plus obidoxime therapy. These effects are comparable to diazepam at 5 mg kg^{-1}. However, diazepam exhibits myorelaxant activity at therapeutic doses, which are only observed at 5–10 times the therapeutic doses of imidazenil.

Keywords: benzodiazepines; convulsions; DFP; imidazenil; organophosphate

1 INTRODUCTION

Centrally mediated seizures are one of the toxic signs following poisoning with organophosphates (OP), Benzodiazepines (BDZ), especially diazepam, are very effective in the management of these convulsions when given as adjuncts to atropine and oxime.[1,2] However, sedation, myorelaxation and dependence make BDZ a poor choice for the treatment in these states. Partial BDZ receptor agonists are regarded as producing these side effects only in very high doses. Some of these drugs, like compound CGS9896, are very effective in the treatment of OP intoxications[3] but unfortunately they are not registered as drugs. The recently reported partial allosteric modulator of BDZ receptors, imidazenil (6-(2-bromophenyl)-8-fluoro-4*H*-imidazo [1,5-*a*] benzodiazepine-3-carboximide),[4] is more like the ideal drug for the management of OP-induced convulsions. This drug is now under extensive studies in many laboratories and clinics and is considered as a potential novel anti-epileptic drug.[5] The present study was performed in order to determine anti-convulsant effects of imidazenil in acute OP intoxications, as well as to establish its antidotal efficacy in rodents.

2 MATERIAL AND METHODS

2.1 Tested drugs

Fluostigmine (diisopropyl phosphorofluoridate; DFP) was used as a model OP compound

2.2 Anti-convulsant efficacy

2.2.1 Effects on convulsion intensity

These were determined on 20 Swiss strain male mice divided in three groups (control and two experimental) using a Convulsometer (Columbus Instruments, USA). The control group received DFP (5 mg kg^{-1} sc) and obidoxime (40 mg kg^{-1} ip) and experimental groups additionally imidazenil (2 mg kg^{-1} ip) or diazepam (5 mg kg^{-1}) immediately after the intoxication. Intensity of subsequent convulsions was measured 10, 30, 60 and 120 min after the treatment and presented in g s^{-1}